Supplemental Information

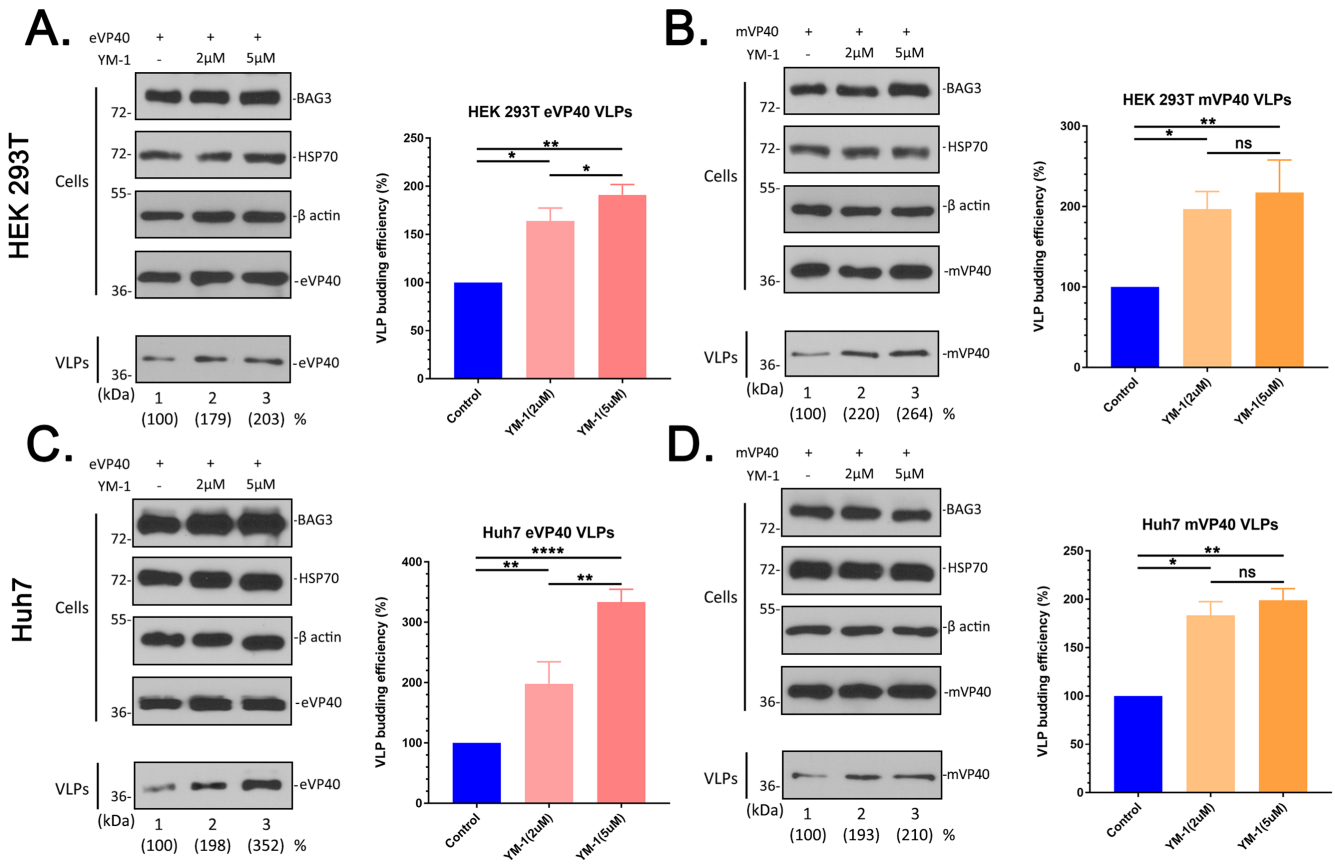
Chaperone Assisted Selective Autophagy (CASA) Targets Filovirus VP40 as a Client and Restricts Egress of Virus Particles

Jingjing Liang[1], Marija A. Djurkovic[2], Olena Shtanko[2], and Ronald N. Harty[1]*

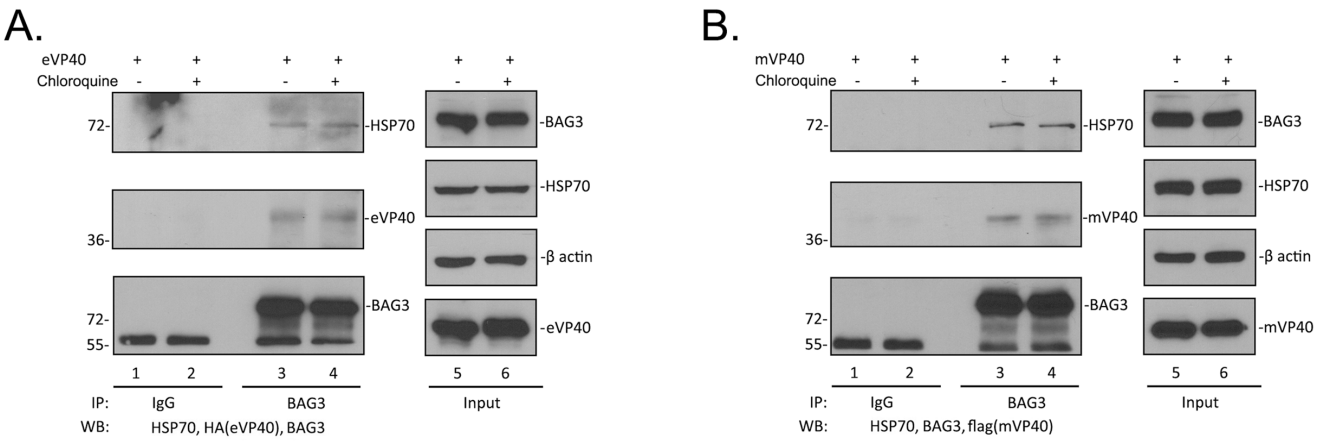
1 Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA. 19104, USA; 2 Host-Pathogen Interactions, Texas Biomedical Research Institute, 8715 W. Military Drive, San Antonio, TX. 78227, USA.

***Corresponding Author**: Dr. Ronald N. Harty, Professor, Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, 3800 Spruce Street, Philadelphia, PA 19104, USA. Phone: 215-573-4485, Fax: 215-898-7887, Email: rharty@vet.upenn.edu

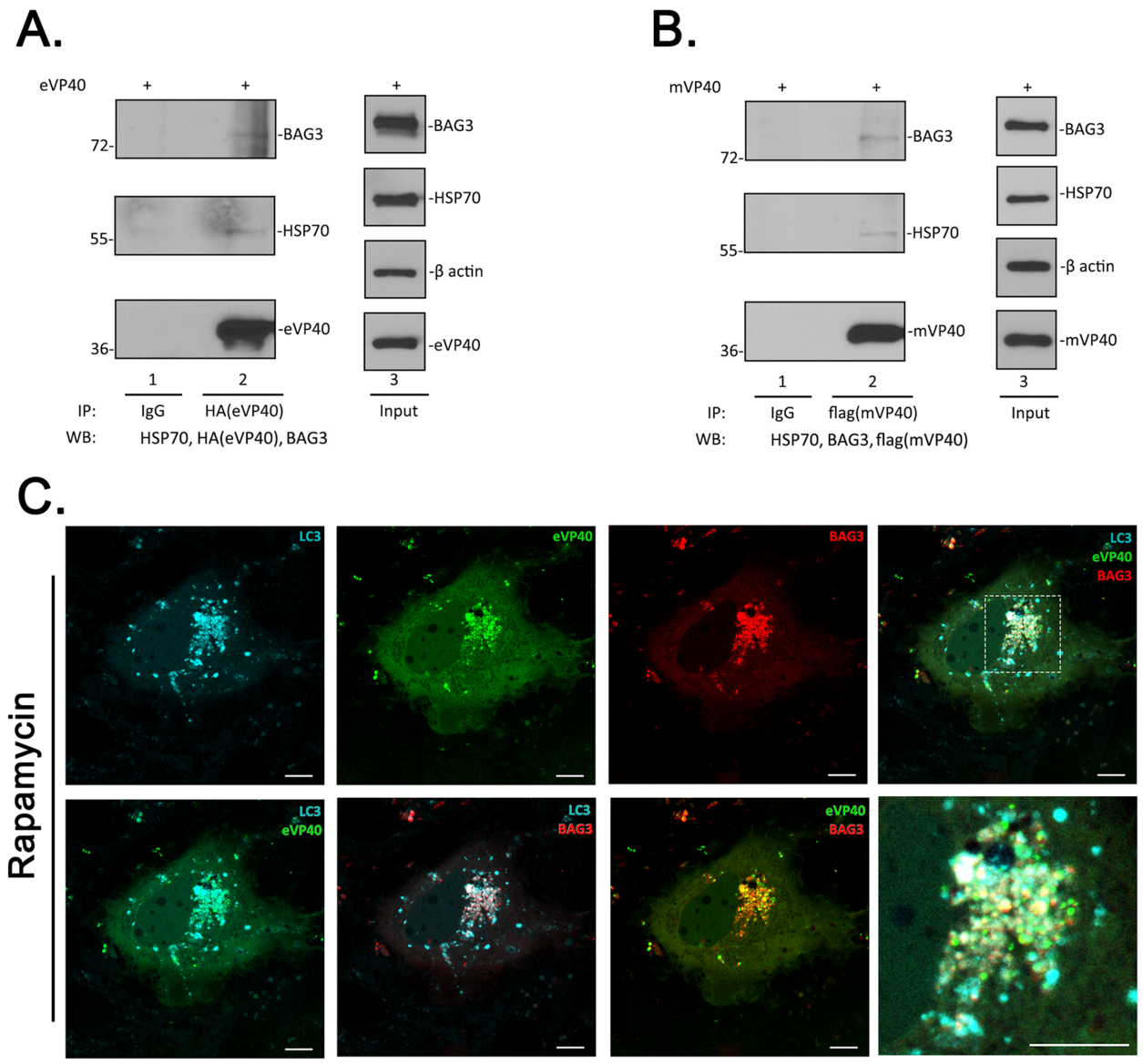
Keywords: filovirus, Ebola, Marburg, VP40, budding, BAG3, autophagy, mTORC1, CASA



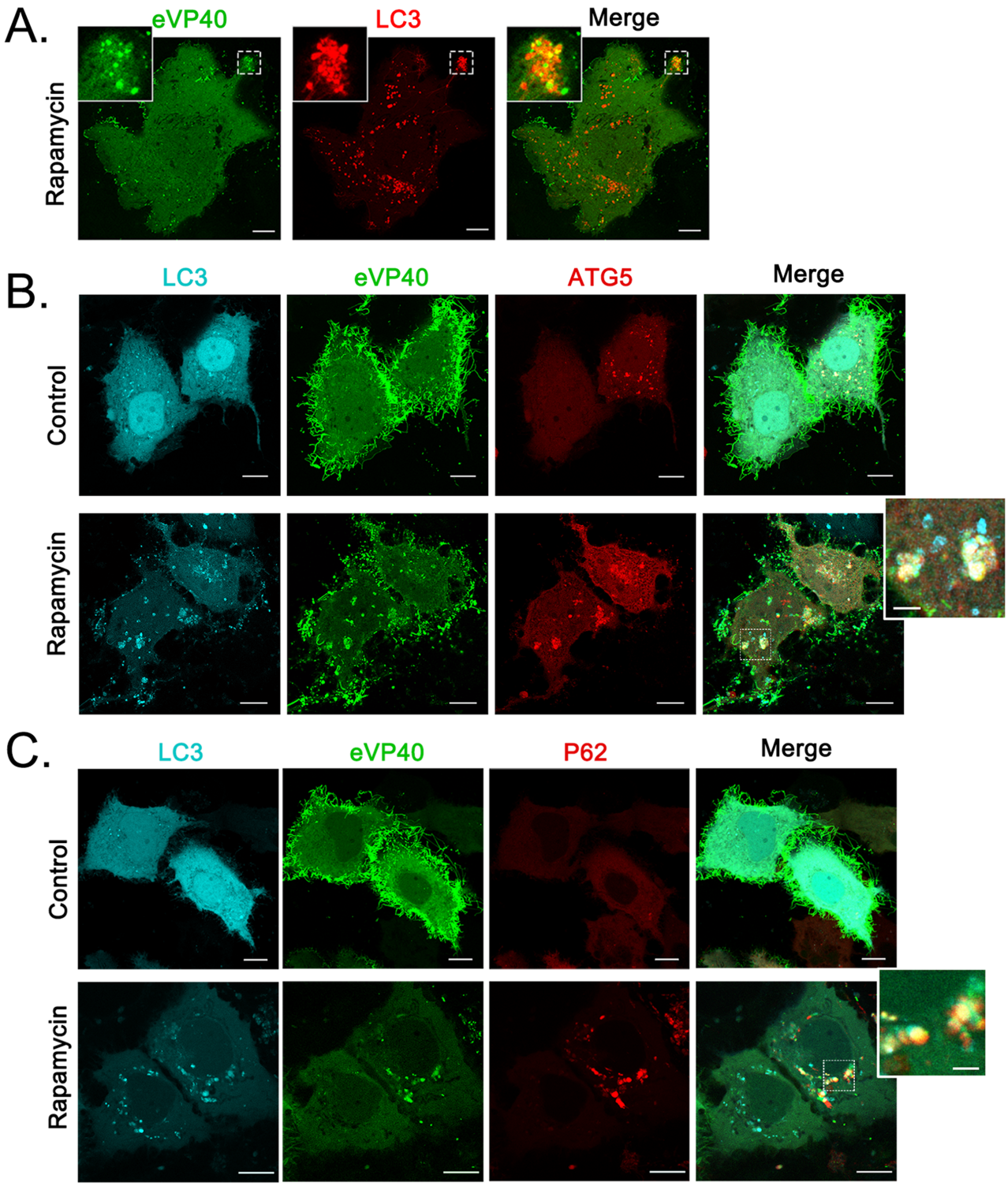
SFig. 1. YM-1 treatment enhances VP40 VLP egress in a dose-dependent manner. Representative eVP40 (**A** and **C**) and mVP40 (**B** and **D**) VLP budding assays and VP40 VLP quantification in the absence or presence of increasing concentrations of YM-1 in HEK 293T cells (**A** and **B**) or Huh7 cells (**C** and **D**). Statistical significance was analyzed by one-way ANOVA. ns: not significant, *=p<0.05, **=p<0.01, ****= p<0.0001.



SFig. 2. The formation of the VP40/BAG3/HSP70 complex with or without CQ treatment. **A and B)** Co-IP assays showing that CQ treatment (10μM) did not affect the interactions among BAG3, HSP70 and eVP40 (**A**) or mVP40 (**B**) detected in the HEK293T cells.

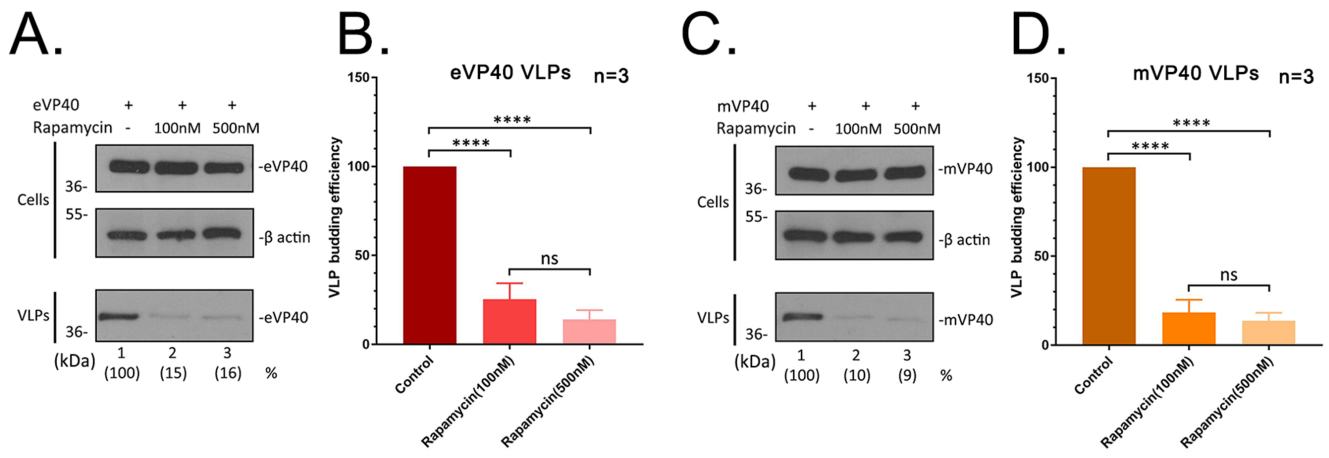


SFig. 3. BAG3/HSP70/VP40 complex formation and rapamycin-mediated delivery of BAG3/VP40 to autophagosomes (APs) in Huh7 cells. Co-IP assays showing the interactions among eVP40 (**A**) or flag-tagged mVP40 (**B**) and endogenous BAG3 and HSP70 in Huh7 cells. **C)** Representative confocal images of Huh7 cells expressing LC3-CFP (cyan), YFP-eVP40 (green) and/or BAG3-mCherry (red) in the presence of rapamycin (200nM). The enlarged inset highlights the colocalization of LC3, BAG3, and eVP40 in clustered APs in the perinuclear region. Scale bars=10μm.

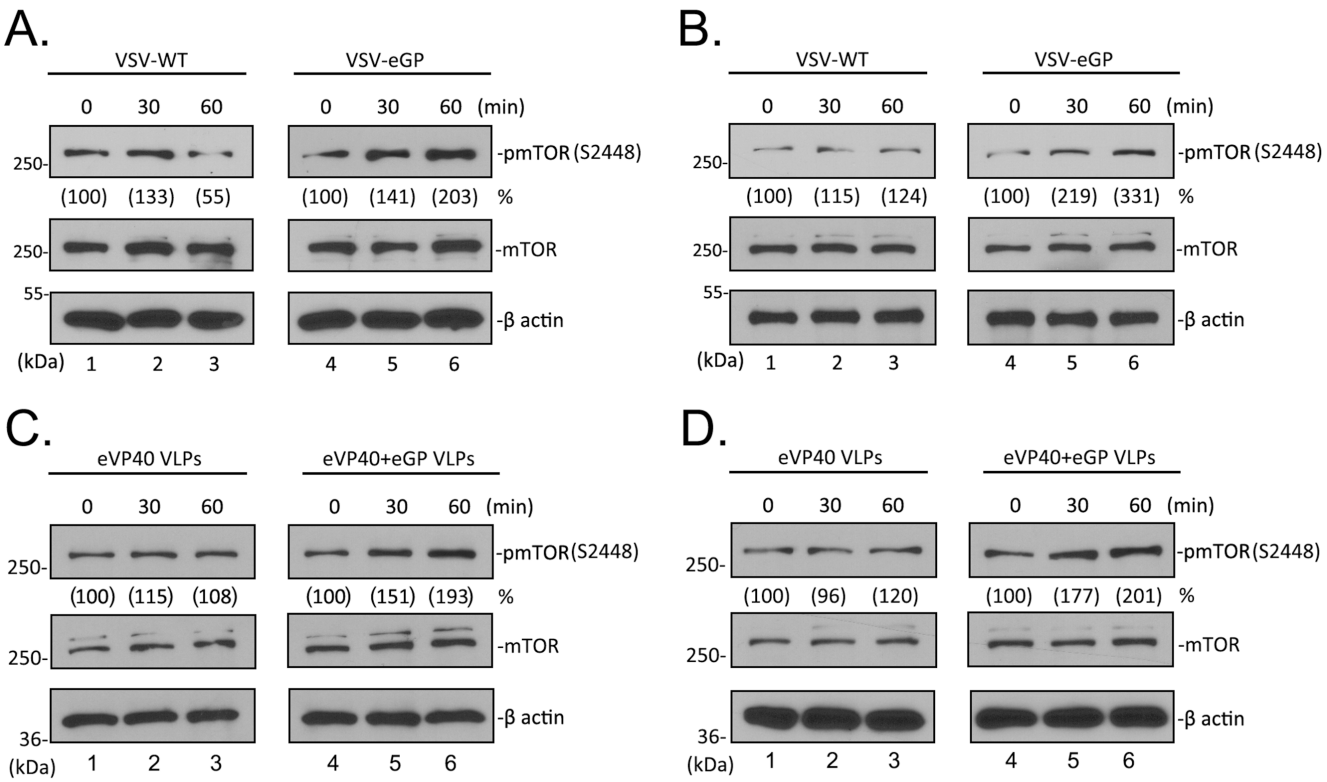


SFig. 4. eVP40 colocalizes with autophagic vesicle markers LC3, ATG5 and P62. **(A)** Confocal images of Huh7 cells showing LC3-marked autophagosomes (red) gathered at the cell periphery

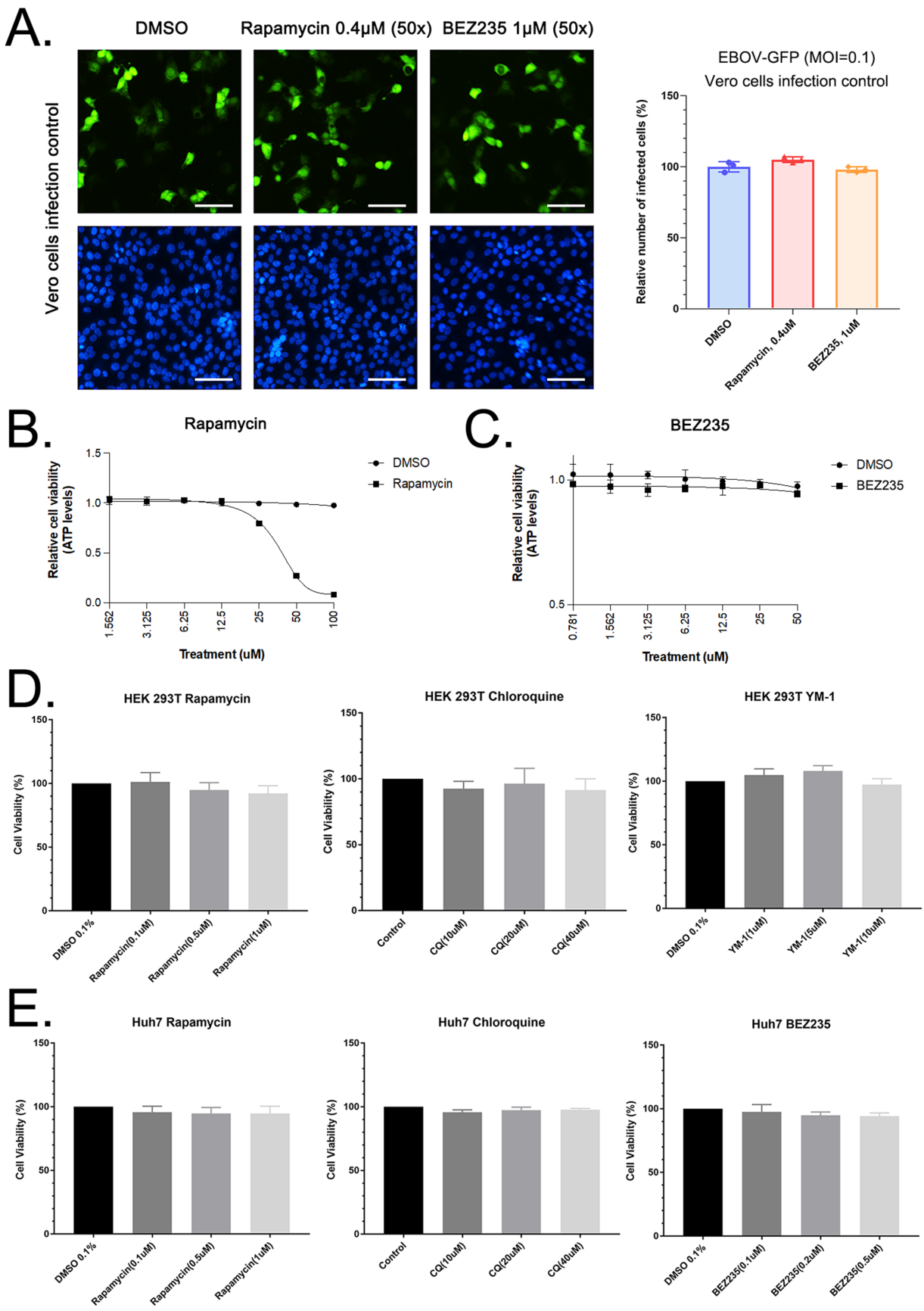
with eVP40 aggregates (green) in the presence of rapamycin (200nM). **B and C)** Confocal images of Huh7 cells expressing the indicated combinations of proteins including LC3-CFP (cyan), YFP-eVP40 (green), ATG5-mCherry (red), and/or p62 (red) in the absence or presence of rapamycin (200nM). The control cells show the typical pattern of eVP40 enriched predominantly in plasma membrane projections (**B** and **C**, top rows), whereas treatment with rapamycin results in a significant shift of eVP40 localization from PM projections to autophagic vesicles as determined by colocalization of eVP40 with LC3 and phagophore membrane proteins ATG5 (**B**, bottom row) or selective autophagy receptor p62 (**C**, bottom row). Scale bars=10μm in main panels and 2μm in insert panels.



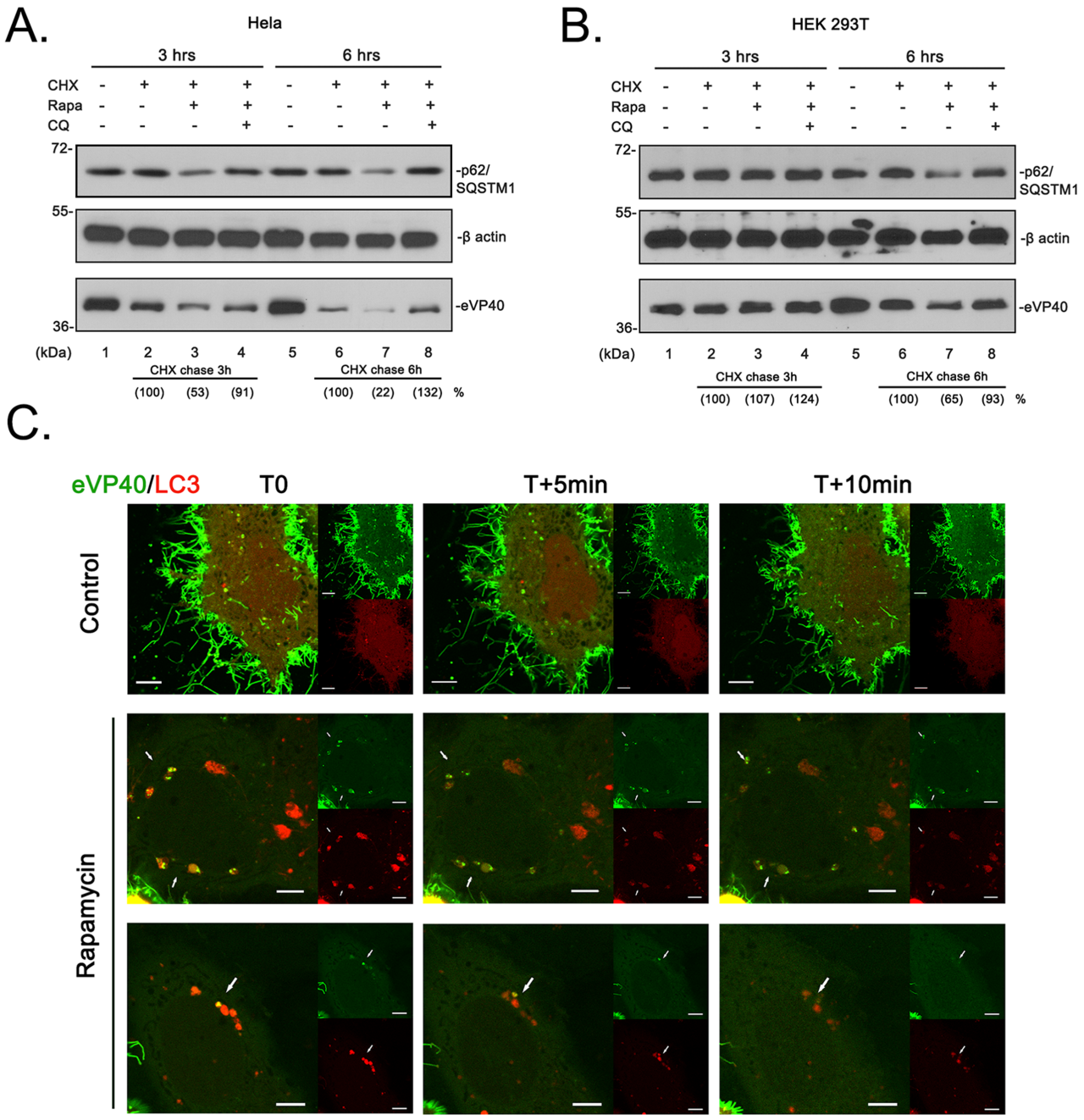
SFig. 5. Rapamycin inhibits VP40 VLP budding in HEK293T cells. **A-D)** Representative eVP40 (**A**) and mVP40 (**C**) VLP budding assays in HEK293T cells and VP40 VLP quantification (**B** and **D**) from 3 independent assays (n=3) in the absence or presence of rapamycin. Statistical significance was analyzed by one-way ANOVA. ns: not significant, ****= $p<0.0001$.



SFig. 6. Surface expression of EBOV GP on recombinant VSV or VLPs activates mTORC1. **A-D)** Independent repeats of Western blot assays (from a total of 3) to detect and quantify phosphorylated mTOR (pmTOR) in Huh7 cells incubated for the indicated amount of time with VSV-WT (**A** and **B**, lanes 1-3), VSV-eGP (**A** and **B**, lanes 4-6), eVP40 VLPs (**C** and **D**, lanes 1-3), or eVP40+eGP VLPs (**C** and **D**, lanes 4-6). Quantification of pmTOR was determined by NIH Image-J and is indicated in parentheses.


A.
DMSO
Rapamycin 0.4μM (50x)
BEZ235 1μM (50x)
Vero cells infection control
EBOV-GFP (MOI=0.1)
Vero cells infection control
Relative number of infected cells (%)
150
100
50
0
DMSO
Rapamycin, 0.4uM
BEZ235, 1uM
B.
Rapamycin
Relative cell viability (ATP levels)
1.5
1.0
0.5
0.0
DMSO
Rapamycin
1.562
3.125
6.25
12.5
25
50
100
Treatment (uM)
C.
BEZ235
Relative cell viability (ATP levels)
1.0
0.5
DMSO
BEZ235
0.781
1.562
3.125
6.25
12.5
25
50
Treatment (uM)
D.
HEK 293T Rapamycin
HEK 293T Chloroquine
HEK 293T YM-1
Cell Viability (%)
150
100
50
0
DMSO 0.1%
Rapamycin(0.1uM)
Rapamycin(0.5uM)
Rapamycin(1uM)
Control
CQ(10uM)
CQ(20uM)
CQ(40uM)
DMSO 0.1%
YM-1(1uM)
YM-1(5uM)
YM-1(10uM)
E.
Huh7 Rapamycin
Huh7 Chloroquine
Huh7 BEZ235
Cell Viability (%)
150
100
50
0
DMSO 0.1%
Rapamycin(0.1uM)
Rapamycin(0.5uM)
Rapamycin(1uM)
Control
CQ(10uM)
CQ(20uM)
CQ(40uM)
DMSO 0.1%
BEZ235(0.1uM)
BEZ235(0.2uM)
BEZ235(0.5uM)

SFig. 7. EBOV infection control and cell viability assays. **A)** Confocal images and quantification (bar graph) of live EBOV-GFP infected Vero cells at 24 h.p.i. in the absence or presence of 50x diluted (corresponding to Huh7 supernatant) concentration of rapamycin or BEZ235. Scale bars=50 μm. **B and C**) ATP cell viability assays were performed on Huh7 cells that were mock treated or treated with the indicated concentrations of rapamycin (**B**) and BEZ235 (**C**) under conditions that mimicked those used for authentic EBOV infection. CCK8 cell viability assays were performed on HEK 293T (**D**) or Huh7 cells (**E**) that were mock treated or treated with the indicated concentrations of rapamycin, chloroquine, YM-1, or BEZ235 under conditions that mimicked those used in VP40 VLP budding assays.



SFig. 8. The autophagic-lysosomal degradation of eVP40. Rapamycin induced the autophagic-lysosomal degradation of eVP40. Representative cycloheximide (CHX; 10 μg/ml) chase assays [1, 2] in Hela (**A**) and HEK293T (**B**) cells showing rapamycin-induced degradation of eVP40 via autophagic flux (compare lanes 2 and 3, and compare lanes 6 and 7). The addition of CQ

blocked the autophagic flux and thus reversed the rapamycin-induced autophagic degradation of eVP40 (compare lanes 2 and 4, and compare lanes 6 and 8). Degradation of eVP40 was more pronounced in Hela cells compared to that in HEK293T cells. As expected, p62 was also degraded via autophagic flux (lanes 3 and 7) and thus served as an internal control. **C)** Representative confocal images from a time-lapse experiment of live Huh7 cells expressing GFP-eVP40 (green) and LC3-mCherry (red) in the absence (top row) or presence of rapamycin (middle and bottom rows). eVP40 becomes sequestered in LC3-marked vesicles and is degraded over time (see accompanying videos S1-S3). Scale bars=5 μm.

Video. S1-S3. Rapamycin induces autophagic sequestration and degradation of eVP40. **(S1)** eVP40 VLP egress in untreated Huh7 cells (control). **(S2)** eVP40 forms aggregates and becomes sequestered in LC3 marked autophagic vesicles in rapamycin treated Huh7 cells. **(S3)** eVP40 aggregates are degraded within LC3 marked autophagic vesicles in rapamycin treated Huh7 cells. Huh7 cells exogenous expressing GFP-eVP40 and hLC3-mCherry were mock treated or treated with rapamycin (200nM) for 14 hours and imaged via confocal microscopy. A total of 100 frames were collected with a time interval of 6s for 10min. Still images are shown in Fig. S8C. The video is displayed as 10 frames per second. Scale bars=5 μm.

Materials and Methods:

Plasmids, antibodies and reagents. Plasmids encoding eVP40, HA-tagged eVP40, GFP-eVP40 and YFP-eVP40 were described previously [3, 4]. Flag-tagged mVP40-WT was kindly provided by S. Becker (Institut für Virologie, Marburg, Germany). The pcDNA6 myc-His-BAG3 plasmid was kindly provided by K. Khalili (Temple University). Plasmid pDEST-mCherry-BAG3 was kindly provided by E. Sjøttem (University of Tromsø), pcDNA3.1-mCherry-hLC3B was a gift from David Rubinsztein (Addgene plasmid # 40827), pEX-CFP-hLC3B was a gift from Isei Tanida (Addgene plasmid # 24985), pDEST-CMV mCherry-GFP-LC3B was a gift from Robin Ketteler (Addgene plasmid # 123230).

Anti-BAG3 (10599-1-AP), anti-HSC70 (HSPA8) (10654-1-AP), anti-HA (66006-2-Ig), anti-mTOR (66888-1-Ig), anti-Phospho-mTOR (Ser2448) (67778-1-Ig), anti-hLC3 (14600-1-AP) antibodies were purchased from Proteintech. Other antibodies used included anti-Flag (Sigma, F1804), anti-β-actin (Sigma, A1978), anti-eVP40 (IBT, 0301-010), anti-HSP70 (CST, 46477), anti-myc (EMD Millipore, 05-724), anti-sodium potassium ATPase (Abcam, ab76020).

Additional reagents included siRNA control (D-001810-10-05), human BAG3 siRNA (L-011957-01-0005) (Dharmacon), YM-1 (Sigma, SML0943), Chloroquine (Sigma, C6628), Rapamycin (MedChemExpress, HY-10219), and BEZ235 (MedChemExpress, HY-50673).

Cells. HEK293T, Huh7, and Vero cells were maintained in DMEM (Corning), HAP1 WT cells (kindly provided by K. Chandran, Albert Einstein College of Medicine) and HAP1-BAG3 KO cells (Horizon Discovery) were maintained in IMDM (Corning), supplemented with 10% FBS (Gibco), penicillin (100U/ml)/streptomycin (100µg/ml) (Invitrogen), and the cells were maintained at 37°C

in a humidified 5% CO2 incubator. The HAP1-BAG3 KO cells contain an 11 base pair deletion in the coding exon of BAG3.

Viruses. VSV-WT (Indiana strain) and VSV-eGP were described previously [5, 6]. All experiments with infectious EBOV were performed in the biosafety level 4 (BSL-4) laboratory at the Texas Biomedical Research Institute, San Antonio, TX. The recombinant EBOV variant Mayinga expressing GFP (EBOV-GFP) (NCBI accession number KF_990213) was grown and titrated as described previously [7]. Briefly, amplified virus was serially diluted and then incubated with Vero cells for 24 hours, then stained with Hoechst 33342 dye (Thermo Fisher Scientific) to identify nuclei, and photographed using Nikon Ti-Eclipse microscope running high-content analysis software (Nikon, Tokyo, Japan). The numbers of GFP-expressing (infected) cells and nuclei were determined by CellProfiler software (Broad Institute).

Immunoprecipitation assay. HEK293T cells were transfected with HA-tagged eVP40 or flag-tagged mVP40 plasmids using Lipofectamine reagent (Invitrogen). At 24 hours post transfection, cells were harvested and lysed. Cell extracts were incubated with either normal mouse or rabbit IgG, and specific antisera as indicated, following by gentle rotating overnight at 4 °C. Protein A/G plus agarose beads (Santa cruz) were then added to the mixtures and incubated 4 hours at 4°C. After incubation, beads were collected via centrifugation and washed 5 times. Proteins were then detected by Western blotting with specific antisera as indicated.

siRNA knockdown. HEK293T cells were transfected with human BAG3-specific or random siRNA (Dharmacon) at a final concentration of 100nM using Lipofectamine 2000 (Invitrogen). At 24 hours post siRNA transfection, cells were transfected again with eVP40 or mVP40 plasmids, and cell extracts were harvested at 24 hours post plasmid transfection for co-immunoprecipitation analysis.

VLP budding assay. Filovirus VP40 VLP budding assays in HEK293T cells were described previously [8]. eVP40 and mVP40 proteins in VLPs and cell extracts were detected by Western blotting and quantified using NIH Image-J software.

Pharmacological assays. HEK293T cells were transfected with eVP40 or mVP40 plus vector or myc-tagged BAG3 plasmids. Cells were mock treated or treated with YM-1(1μM and 5μM) for 16 hours. Cell extracts were harvested for co-IP analysis, or cell extracts and supernatants were harvested for Western blotting and VLP budding analysis. Cells were mock treated or treated with CQ (10μM or 20μM) for 16 hours. Cell extracts and supernatants were harvested for co-IP analysis, Western blotting or VLP budding analysis, or cells were harvested for subcellular protein fractionation.

Cytosol and plasma membrane protein fractionation. HEK293T cells were collected via low-speed centrifugation. The nuclear, cytosol, organelle membrane and plasma membrane protein fractions were isolated sequentially using the plasma membrane protein isolation and cell fractionation kit (INVENT) following the manufacturer's instructions. Proteins within the cytosol and plasma membrane fractions were analyzed via Western blotting. The β-actin and sodium potassium ATPase were used as a cytosol and plasma membrane loading controls, respectively.

Live cell imaging. Huh7 cells were transfected with YFP-eVP40 and hLC3B-CFP plus vector alone or BAG3-mCherry plasmids. Cells were monitored via confocal microscopy at 14 hours post-transfection. Huh7 cells were transfected with indicated plasmids combinations (GFP-eVP40/hLC3B-mCherry, YFP-eVP40/hLC3B-CFP/p62-mCherry, or YFP-eVP40/hLC3B-CFP/ATG5-mCherry) and mock treated or treated with rapamycin (200nM) for 14 hours, and then monitored via confocal microscopy. Cell nuclei were stained by NucBlue™ Live

ReadyProbes™ Reagent (Invitrogen). Images were obtained on a Leica SP5 inverted confocal microscope with a 100x (NA 1.46) objective lens using sequential scanning.

mTOR inhibitors and VLP budding assay. Huh7 cells were transfected with eVP40 or mVP40 using Lipofectamine 2000 (Invitrogen). After 8 hours, cells were mock treated or treated with rapamycin (100nM and 200nM) or BEZ235 (100nM and 200nM) for 16 hours. Cell extracts and supernatants were harvested for Western blotting and VLP budding analysis. The membranes used to detect the phosphorylated (S2448) mTOR levels were stripped and re-probed for detection of the total mTOR levels.

Recombinant virus and VLP incubation. Huh7 cells were incubated with VSV WT or recombinant virus VSV-eGP-mCherry at an MOI=3.0, or with purified VLPs containing eVP40 alone or eVP40+eGP resuspended in DMEM at 37°C. Inoculums were removed and cells were washed and lysed at the indicated time points. Cell extracts were analyzed by Western blotting. VLPs were produced in HEK 293T cells.

EBOV infection assays. To determine a nontoxic concentration range of rapamycin and BEZ235 treatments for virus inhibition studies, Huh7 cells grown in 96-well plates were left untreated or treated with either compound at seven 2-fold serially diluted concentrations or DMSO for 24 hours. All treatments were performed in triplicate. The number of metabolically active cells was determined using a CellTiter-Glo 2 reagent. The concentration that reduced the cell viability by <95%, as compared to the untreated cells, was determined by GraphPad 8 softare and used as the highest treatment concentration in virus assays. The lower concentration was used to determine dose-dependent virus response.

To test whether rapamycin and/or BEZ235 affect EBOV egress, Huh7 cells seeded in wells of a 96-wells plate were incubated with EBOV-GFP at an MOI of 0.1, in triplicate. After 10 hours,

virus was removed and cells washed with warm medium. Fresh medium containing indicated concentrations of the compounds or an equal amount of DMSO was added onto cell monolayers for 14 hours. Cell supernatants were titrated onto Vero cells for 24 hours to determine release of infectious virus. All cell monolayers were fixed in 10% buffered formalin solution in accordance with Institutional SOPs and approved Biohazard and Saftey Committee protocols to inactivate virus. Subsequently, cells were stained with Hoechst 33342 dye and analyzed as above. Infection efficiency in all samples was determined as a ratio of GFP-positive (infected) cells and nuclei and reported relative to the values in DMSO control. Statistical significance for the rapamycin and BEZ235 treatments was calculated relative to DMSO control using ANOVA with Tukey's multiple comparison test.

Monitoring autophagic flux in live cells. Huh7 cells exogenously expressing the tandem fluorescent tagged hLC3B were mock treated or treated with rapamycin (200nM) or CQ (10μM) for 14 hours, and then were monitored via confocal microscopy on a Leica SP5 inverted confocal microscope with a 100x (NA 1.46) objective lens using sequential scanning.

Autolysosome tracking in live cells. Huh7 cells exogenously expressing GFP-eVP40 and hLC3B-mCherry were mock treated or treated with rapamycin (200nM) or rapamycin (200nM) plus with CQ (10μM) for 14 hours, and then cells were incubated with grown medium containing 75nM LysoTracker™ Deep Red (Invitrogen) for 1 hours at 37 °C. The probe-containing medium was replaced by fresh medium, and cells were monitored via confocal microscopy on a Leica SP5 inverted confocal microscope with a 100x (NA 1.46) objective lens using sequential scanning.

Cycloheximide chase assays. Huh7, Hela and HEK293T cells exogenously expressing eVP40 were mock treated or treated with cycloheximide alone (10μg/ml), or in combination with

rapamycin (1μM), or rapamycin (1μM) plus CQ (10μM) at 20 hours post transfection. Cell extracts were harvested at 3h or 6h after treatment for Western blotting analysis. The cellular eVP40 levels during CHX chase were quantified using NIH Image-J software.

Statistical analysis. Significances were calculated from three independent experiments in GraphPad Prism by either One-way ANOVA-Dunnett's T3 multiple comparisons test, or Unpaired t-test with Welch's correction as indicated in the legend.

References:

1. Zhao, J.H., et al., *mTOR inhibition activates overall protein degradation by the ubiquitin proteasome system as well as by autophagy.* Proceedings of the National Academy of Sciences of the United States of America, 2015. **112**(52): p. 15790-15797.
2. Sha, Z., J. Zhao, and A.L. Goldberg, *Measuring the Overall Rate of Protein Breakdown in Cells and the Contributions of the Ubiquitin-Proteasome and Autophagy-Lysosomal Pathways.* Methods Mol Biol, 2018. **1844**: p. 261-276.
3. Liang, J., et al., *Angiomotin Counteracts the Negative Regulatory Effect of Host WWOX on Viral PPxY-Mediated Egress.* J Virol, 2021.
4. Liang, J., et al., *Chaperone-Mediated Autophagy Protein BAG3 Negatively Regulates Ebola and Marburg VP40-Mediated Egress.* PLoS Pathog, 2017. **13**(1): p. e1006132.
5. Liang, J.J., et al., *WWOX-Mediated Degradation of AMOTp130 Negatively Affects Egress of Filovirus VP40 Virus-Like Particles.* Journal of Virology, 2022. **96**(6).
6. Han, Z.Y., et al., *Ebola virus mediated infectivity is restricted in canine and feline cells.* Veterinary Microbiology, 2016. **182**: p. 102-107.
7. Shtanko, O., et al., *Retro-2 and its dihydroquinazolinone derivatives inhibit filovirus infection.* Antiviral Res, 2018. **149**: p. 154-163.
8. Han, Z., et al., *Modular mimicry and engagement of the Hippo pathway by Marburg virus VP40: Implications for filovirus biology and budding.* PLoS Pathog, 2020. **16**(1): p. e1008231.